



APPENDIX A

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(21) International Application Number: PCT/US99/06762 (22) International Filing Date: 29 March 1999 (29.03.99) (30) Priority Data: 60/079,935 30 March 1998 (30.03.98) US (71) Applicant (for all designated States except US): ESA, INC. [-/US]; 22 Alpha Road, Chelmsford, MA 01824 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KRISTAL, Bruce, S. [US/US]; Apartment 101, 801 Mamaroneck Avenue, White Plains, NY 10605 (US). MATSON, Wayne, R. [US/US]; One Harvard Road, Ayer, MA 01433 (US). MILBURY, Paul, E. [US/US]; 17 Skytop Road, Ipswich, MA 01938 (US). (74) Agent: SOLOWAY, Norman, P.; Hayes, Soloway, Hennessey, Grossman & Hage, 175 Canal Street, Manchester, NH 03101 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHODOLOGY FOR PREDICTING AND/OR DIAGNOSING DISEASE (57) Abstract Disorders are diagnosed by analyzing biological samples of ad libitum-fed and dietary-restricted individuals to generate frequency distribution patterns representative of molecular constituents of the samples, and comparing the patterns.		

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1 **METHODOLOGY FOR PREDICTING AND/OR DIAGNOSING DISEASE**

2 **Field of the Invention**

3 This invention in one aspect relates to a method for predicting and/or
4 diagnosing diseases in living animals. The invention has particular utility in
5 diagnosing and/or predicting future risk of specific diseases in living animals and will
6 be described in connection with such utility, although other utilities are contemplated.
7 This invention in another aspect relates to identification of markers for diseases or
8 sub-clinical conditions that in the future may develop into diseases that are capable of
9 distinguishing groups, and to subsets of these markers, where the utility of such
10 markers can, for example, be determined by univariate, multivariate, or pattern
11 recognition based analyses, and/or where the markers identified as important by the
12 approach described also can be measured using other analytic approaches. The
13 invention has particular applicability to predicting risk to cancer, type II diabetes,
14 cardiovascular disease, cerebrovascular disease, and other diseases whose etiology has
15 been established to or hypothesized to be modified by diet or nutrition, i.e.
16 neurogenerative disorders such as Alzheimer's Disease, Parkinson's Disease and
17 Huntington's Disease {1}, and will be described specifically in connection its utility
18 for using serum or plasma metabolites for determining breast cancer risk; however,
19 other utilities and other tissue or biological fluid samples (e.g., whole blood,
20 cerebrospinal fluid, urine, and/or tissue samples) may be used instead of blood, and
21 diseases and conditions other than breast cancer also can be addressed, as noted
22 above. Similarly, in addition to disease, the assessment of nutritive status (over long
23 or short term), may be utilized in accordance with yet another aspect of the present
24 invention as a medical test under a variety of potential clinical settings, or in
25 controlling epidemiological or pharmaceutical testing. Still other utilities, e.g. for
26 detecting exposure to and/or sensitivity to exposure to toxins, are contemplated.

27 **Background of the Invention**

28 Dietary restriction (DR), i.e. underfeeding without malnutrition, has
29 established efficacy in reducing both degenerative and neoplastic diseases. DR has
30 been extensively explored since its first use in the 1930's because of its ability to
31 extend both mean and maximum life span, reduce age-related morbidity, and delay or

1 prevent certain age-associated physiological dysfunction {2, 3}. DR also alters many
2 basic physiological processes, including metabolism, hormonal balance, and the
3 generation of, detoxification of, and resistance to reactive oxygen species {4}. DR
4 can be implemented in multiple ways {e.g. 5-13}. Moreover, restriction of total
5 calories is believed to be more important than reducing intake of specific factors (e.g.
6 fat, proteins, vitamins and minerals, etc. {14, 15}). DR reportedly extends longevity
7 in essentially all animals in which it has been tried, including multiple mammalian
8 species (rat, mouse, guinea pig {2, 5-13, 16}). Furthermore, promising data suggest
9 that at least some of the benefits of DR, especially those regarding glucose
10 metabolism, also occur in non-human primates {17-21}, and perhaps, in humans as
11 well {22,23}. Together, these observations suggest that the DR effect is robust in
12 mammals.

13 DR has been shown to reduce both incidence and severity of non-neoplastic
14 diseases. One example is the efficacy of DR against glomerulonephritis, periarteritis,
15 and myocardial degeneration in both male and female Sprague-Dawley rats. Similar
16 observations have been made in other strains and other diseases, such as lung disease
17 {25}. DR is also effective at preventing some strain specific disease, such as auto-
18 immune disease in NZB/NZWF1 mice {26} and in MRL/lpr mice {27}, and
19 atherosclerotic {28} and myocardial ischemia lesions in JCR:LA-cp mice {29}.

20 DR also has been shown to reduce both incidence and severity of neoplastic
21 diseases. DR-mediated reduction of neoplasia includes delayed onset of leukemia,
22 pituitary adenomas, mammary and prostatic tumors, and hepatomas {30, 31}.
23 Observations of the effects of DR on mammary tumors {32-36} are typical. DR acts
24 to reduce breast cancer both by delaying onset (both by reducing initiation events and
25 slowing promotion) and by slowing tumor progression {30}. In transgenic mice prone
26 to mammary tumors, DR reduced tumor incidence by 67% {32}. This result reveals
27 that DR is capable of overcoming genetic predisposition to breast cancer. Studies
28 {33} in rats treated with a carcinogen demonstrated that high fat and high calorie diets
29 are co-carcinogenic, and that *none* of the rats maintained on 40% DR regimen
30 developed mammary tumors, while 60% of AL-fed rats did. Concerns that this effect
31 may have been partially mediated by reducing fat availability for tumor growth led to

1 later studies {34}. Despite a higher fat content in the DR diet, results show a 75%
2 reduction in rats with mammary tumors and in the number of tumors per animal in the
3 tumor-bearing group. Even more impressively, DR reduced total tumor yield, average
4 tumor size, and mean tumor burden by 93-98%. Notably, Sinha et al demonstrated
5 that even a 20% DR regimen reduces tumors by 65%, without effects on hormone
6 levels or fertility {35}.

7 Thus, DR mediated protection against breast cancer in laboratory models is:
8 1) substantial (as much as 100% reduction in cancer rates {32}) and highly replicable
9 {30-34}; 2) robust and well-documented in a variety of animal models, including a
10 model of genetic predisposition and a model of carcinogen exposure {31, 32}; 3)
11 seen even with a more moderate (20%) restriction paradigm that does not affect
12 fertility or hormone levels {34}; 4) effective at multiple levels (initiation, promotion,
13 progression). Thus, the present invention, in one aspect, is based on the observation
14 that different subsets of markers that reflect DR are predictive for different diseases.
15 For example, identifying markers, for example in sera, that reflect the DR phenotype,
16 would lead to markers that would reflect risk of developing breast cancer, or other
17 conditions affected by diet.

18 Consistent with its broad effects on longevity and disease, DR is a systemic
19 phenomenon, and its effects include measurable differences in blood constituents
20 relative to those seen in ad libitum fed (AL) animals {37}. Many previous studies
21 have focused on measurement of hormones. For instance, studies have shown
22 alterations in plasma corticosterone patterns and levels {38}; some female
23 reproductive hormones {39}, plasma cholecystokinin decreases 50% {40}; T3 but not
24 T4 is reduced {41}; and plasma insulin drops as much as 60% in some DR models
25 {42}. While informative, these studies have been somewhat limited by the technical
26 complexity involved (e.g. circadian cyclicity, rapid response to stimuli). Other studies
27 seeking more stable markers have examined markers of energy and free radical
28 metabolism, revealing that DR decreases plasma glucose, ascorbate (e.g. 43-45) and
29 glycohemoglobin levels {43}. Overall, the data indicates that differences in serotype
30 distinguish AL and DR animals, and that these differences include some metabolites
31 that are both relatively easy to assay and which reflect the beneficial effects of DR on

1 physiology, metabolism and free radical biology (e.g. generation, sensitivity, and
2 detoxification).

3 While not wishing to be bound by theory, since the AL and DR serotypes
4 reflect robust physiological differences between these groups, it is believed that these
5 serotypes include metabolites or metabolite profiles that cross-species and predict
6 relative risk for the development of disease in humans. Data consistent with this
7 concept comes from studies showing that the effect of DR on breast cancer is largely
8 driven by chronic effects (termed promotion) rather than acute effects (termed
9 initiation {30, 31}). These data would imply that relative risk of developing breast
10 cancer is likely reflected in general metabolism over long periods of time. Relative
11 risk should thus be detectable in sera long before the development of overt disease. In
12 the case of humans, who lie on a broad spectrum with respect to caloric intake, it is
13 believed that closer fit to the AL serotype (i.e. the biological response typical of a high
14 caloric intake) would predict higher relative risk of disease, whereas greater fit to the
15 DR serotype (i.e. the biological response typical of a lower caloric intake) would be
16 associated with reduced risk. While previous studies demonstrated differences
17 between AL and DR animals, they were believed only able to look at specific,
18 predetermined markers, making it essentially impossible to conduct a sufficiently
19 broad and powerful search to identify markers of use for determining nutritional status
20 or predicting health across species.

21 Summary of the Invention

22 The present invention provides a system, i.e. method and apparatus, for
23 determining differences in concentrations of molecules, in particular small molecule
24 metabolites, between animals whereby to create a metabolite database which may be
25 used to reproducibly distinguish between two or more states of the health or the
26 nutritive status of an animal. More particularly, the present invention employs
27 analysis techniques to provide a small molecule inventory for metabolic pathway
28 patterns of samples of ad libitum fed (AL) and dietary restricted (DR) individuals
29 whereby to reproducibly distinguish between different dietary status of animals,
30 between health conditions of animals, and to reproducibly predict relative risk for the
31 development of a particular disease in animals.

9 Description of the Drawings

26 Detailed Description of Preferred Embodiment

28 Sample preparation:

5

1 were placed on ice for 30 minutes, centrifuged, and the resulting sera snap frozen in
2 liquid nitrogen and stored at minus (-) 80°C until analysis.

3 Samples were precipitated and extracted in four vol of acetonitrile(An)/0.4%
4 acetic acid(HAc) at -20°C. One ml of centrifuged supernatant was removed,
5 evaporated to dryness under vacuum, and reconstituted in 200 µl of a Mobile Phase A
6 as described below. This protocol conserves reactive species such as ascorbate, and
7 homogentistic acid at 1 ng/ml concentrations. 100 µl reconstituted extract was placed
8 in each of two auto sampler vials, one immediately analyzed and the other frozen at -
9 80°C for future confirmation analysis. Prior to injection, samples were maintained at
10 4°C.

11 Mobile Phases: Chromatographic solvents include isopropyl alcohol,
12 methanol, acetonitrile, lithium hydroxide, glacial acetic acid, and pentane sulfonic
13 acid. To retain stability of retention times and response potentials, a novel mobile
14 phase pair was developed: Mobile Phase A (11 g/l of PSA at pH 3.00 with acetic
15 acid) and Mobile Phase B (0.1M LiAc at pH 3.00 with acetic acid in 80/10/10
16 methanol/An/ isopropanol). PSA demonstrates an improved ability to solubilize and
17 remove protein and peptide fragments from both HPLC (C18) columns and
18 coulometric detectors while the high organic modifier (Mobile Phase B) effectively
19 removes residual lipids and polysaccharides. Sulfonic acids are, however, inherently
20 contaminated necessitating a cleaning protocol in which the PSA/HAc concentrated
21 buffer (4 l of 400g/l PSA) was electrolyzed over pyrolytic graphite at a potential of
22 1000 mV vs Pd(H).

23 Chromatographic Methods: Referring to Fig. 1, the chromatographic method
24 involves a 120 min complex gradient from 0% Mobile Phase B to 100% Mobile Phase
25 B, with flow rate adjusted to compensate for aziotropic viscosity effects. Gradient
26 operation was provided by two Shimadzu LC-10AD HPLC pumps. Despite
27 meticulous precleaning protocols, and the use of highly purified solvents and selected
28 organic modifiers, spurious peaks occur late in the gradient. This problem was
29 addressed by developing a device based on electrochemically activated porous carbon
30 with sorption characteristics similar to C18. A prototype peak suppressor/gradient
31 mixer (PS/GM) was placed in stream before the HPLC injector. The PS/GM mixer

1 incorporated a 2 cm length of a 1 cm diameter C18 precolumn integral with a 2.5 cm
2 section of rod with flow interrupting grooves that serve to trap and spread mobile
3 phase contaminants. When these were released to the grooved section, during the
4 gradient run, they were mixed to a peak width at a half height of ca. 140 sec. This
5 effectively reduced a mobile phase derived contaminant signal to a wave that was later
6 eliminated during data reduction. The mixed gradient was delivered from the PS/GM
7 to a PEEK lined pulse damper prior to flowing through the auto sampler injector and
8 on to the C18 columns. Sample extracts were separated on dual PTFE lined HR80
9 columns containing 3-mm ODS particles and measuring 80 mm x 4.6 mm I.D.

10 Analyte detection was accomplished with a NCA Chemical Analyzer, Model
11 CEAS multiple electrode electrochemical detection system, available from ESA, Inc.,
12 of Chelmsford, Massachusetts. The latter includes an ESA Model 6210 analytical cell
13 and a 16-channel coulometric electrode array incremented from -100mV to +940mV
14 to detect both reducible and oxidizable compounds. PS/GM, pulse damper, columns,
15 and detectors are contained within a temperature controlled enclosure maintained at
16 35°C. System functions were controlled by the ESA, Inc. Model 4.12C CEAS
17 software installed on a 386 microcomputer networked to remote 486-based computers
18 where data storage, reduction and analysis were accomplished. CEAS analysis
19 software-produced reports were imported to spreadsheet/database software for further
20 statistical analysis and reports.

21 Data Reduction, Observation and Analysis: Chromatographic retention times,
22 monitored by pure standards and identified sample compounds, do not vary more than
23 1%. The absolute qualitative channel ratio responses do not vary by more than 20%
24 and were controlled for by inclusion of authentic standards to within 5%. Where
25 possible, sample chromatographic peak identities were confirmed by spiking with the
26 relevant authentic standard. Final confirmation was made by comparison of the
27 matching ratio (*R*) of the standard and the sample peaks. *R* represents the ratio
28 between the dominant oxidation channel and juxtaposition subdominant channels. A
29 given compound is oxidized at a specific potential and therefore any compound can be
30 described by a retention time and a potential. In practice, compounds were oxidized
31 on a dominant detector set near its oxidation potential and exhibited a smaller

1 response on the prior and following detector. The ratio exhibited between the
2 dominant and adjacent detector responses was characteristic of a given compound and
3 variations from that ratio, when a standard was close in concentration to a sample
4 compound, indicated a co-eluting contaminant.

5 Data from each detector analog signal was converted and combined with other
6 detector data to construct a time-potential map, which was compared with standards
7 and between samples. Analytical values were calculated for sample peaks based on
8 matches under restrictions for retention time, detector channel ratios and, to a lesser
9 degree, peak heights, according to priority optimized by the analyst over sequential
10 monitored analysis. Where compound identity is known, final results were calculated
11 as ng per ml of sample based on standard responses.

12 To automate analysis, a compound table was generated from a pool of multiple
13 samples in a cohort with concentrations defined as 100. Subsequent sample analysis
14 generates reported values as percentage of pool values. This table was used to analyze
15 (initially with manual oversight, then automatically) all other pools and a few samples
16 within the study. The CEAS analytical software has a built in "learning" capacity,
17 which is inherently part of the "standards" definition function of the analysis. As the
18 operator oversaw a few analyses, decisions were made about parameters such as
19 referencing retention times to other compounds or what degree of variation from the
20 channel ratio's will be tolerated. Conflicts and ambiguity in analysis were monitored
21 and resolved during this test phase of the analysis. Eventually, the pool standard table
22 will "learn" how reliably to find a majority of the potential analytes in the samples.
23 Typically >400 compounds were resolved in plasma at the 20 nanoampere gain.
24 Reported values were captured in a file suitable for downloading into a database.

25 Example I

26 The use of complex HPLC separations, coupled with coulometric array
27 detectors, enables simultaneous quantitation of >400 compounds from serum (Figure
28 2A). The combination of retention time (Figure 2B) and ratio of response across
29 adjacent detectors (Figure 2C) in the array enables reproducible identification of a
30 given peak in multiple runs and comparison of samples of interest such as sera from
31 AL and DR rats. In all, ~70 biochemically identified compounds and 350+ currently

1 unidentified compounds were reproducibly measured using these techniques. See
2 Table I, Fig. 3.

3 **HPLC separations coupled with coulometric array detection**

4 Data was initially generated by CEAS/Coularray systems in the form of a set
5 of 16 chromatograms (one for each detector). Figure 2A shows approximately one-
6 fifth of a total chromatogram, including ~70 independent, identifiable and quantifiable
7 peaks, from a 6-month old male Fischer 344 rat. Sensor potentials ranged from T₁, -
8 100 mv to T₁₆+940mv. Results were shown at an intermediate gain (200 nA). The x
9 axis is retention time, y-axis is the magnitude of the response, the 16 parallel traces
10 represent the 16 detectors of the array from 1-16 (bottom to top). Figure 2B shows a
11 later section of the chromatogram from 3 AL rats (top three traces) and 3 DR rats
12 (bottom three traces). For clarity, only data from channel (detector) 8 is shown (gain
13 = 500 nA). Arrows indicate two metabolites that are decreased by DR. Figure 2C
14 shows the region of the chromatogram from Figure 2A (compound 123, see Figure 4)
15 from one AL (top) and one DR (bottom) animal (gain 15 uA). As in Figure 2A, the
16 16 parallel traces represent the 16 detectors of the array from 1-16 (bottom to top).
17 Note that the ratio of response across the detectors is constant.

18 Application of this technology to the study of sera from AL and DR rats has
19 revealed 34 compounds that differ between these groups (Figure 4). Of these 34
20 compounds, 6 are reproducibly altered in both 6 and 12 month rats, and at least five of
21 these six are also altered in 18 month rats. The remaining 28 markers include some
22 with apparent age-specificity and others whose validity is still under investigation.
23 These markers, which were originally identified in 6-month old AL and DR rats,
24 differ sufficiently between AL and DR groups to separate animals into the correct
25 dietary group by both hierarchical cluster analysis and principal component analysis
26 (Figure 5A and 5B).

27 To verify feasibility, the HPLC system described above was used to determine
28 the relative levels of 217 metabolites from the sera of 6 month old male AL and DR
29 Fischer 344 rats. Analysis revealed 22 metabolites that differed between AL and DR
30 rats by t-test without consideration for the Bonferroni correction (See Figure 4).
31 These 22 compounds (see Table II, Figure 6) became the primary variables of interest

1 in a follow-up study (N=8/group, 12 month AL and DR Fischer 344 rats). Analysis of
2 these data confirmed statistical significance of 6 of these 22 compounds (marked by
3 asterisks in Figure 4). Furthermore, five of these six also statistically differ between
4 18 month old AL and DR rats (p values <0.02, <0.002, <0.001, <0.0002, <0.0001);
5 the sixth (metabolite #71 which was determined to be homovanillic acid) showed a
6 similar trend, but $p > 0.05$ ($\beta < 0.1$, suggesting increasing "N" likely will yield statistical
7 significance). The remaining 16 compounds, as well as 12 compounds that were
8 statistically significant only in the 12 month samples, likely included some that are
9 type I statistical errors, some that may be statistically significant when "N" is
10 increased (β currently <0.8 for many, some of which approach statistical significance
11 in the second age group), and some metabolites may only reflect the DR phenotype at
12 specific ages. Further experiments using the methods described can be used to
13 distinguish between these possibilities, and also to identify other markers of interest.
14 Also, another compound was found to decrease >99% following short term caloric
15 restriction.

16 As will be seen from the foregoing Example, alteration of the dietary paradigm
17 on which animals are maintained can be used to develop specialized patterns or
18 profiles. As examples, tests of male and female rats of different ages enable
19 identification of age- and sex-dependent and -independent profiles associated with
20 DR. Specific changes in the duration and extent of DR feeding regimens enable
21 generation of an extended metabolic database relating markers to long- and short-term
22 caloric intake and balance.

23 Similarly, the resulting data can be analyzed using univariate statistics (e.g., t-
24 tests), multivariate statistics (e.g., ANOVA) or other multivariate analysis
25 (hierarchical cluster analysis, principal component analysis) or through the use of
26 pattern recognition algorithms to qualitatively and quantitatively identify metabolic
27 profiles and relationships.

28 **Serum Markers for DR**

29 Referring to Figure 4, sera samples from male Fischer 344 rats were run on an
30 ESA Model CEAS as described above. Sera from 6-month old and 12 month old AL
31 and DR rats were analyzed (N= 8/group). Data was expressed as the percentage of the

1 level of analyte present in the sera of one of the 6-month old AL rats. Bars to the left
2 of the vertical line represent compounds that differ statistically between 6 month old
3 AL and DR rats; those bars to the right represent compounds that differ statistically
4 between 12 month old AL and DR rats. Asterisks mark the 6 compounds that differ
5 statistically in both groups (bars show only 6 month data; p values below are the value
6 at 6 months). Out of 217 analytes quantified to date, 34 show p values <0.05 prior to
7 Bonferroni corrections, (uncorrected p values, in order {left of line} $p \leq 0.0008$,
8 0.0008, 0.001, 0.001, 0.005, 0.0073, 0.0089, 0.0091, 0.012, 0.012, 0.013, 0.014,
9 0.017, 0.017, 0.017, 0.019, 0.023, 0.026, 0.026, 0.037, 0.04, 0.05; {right of line} $p \leq$
10 0.0017, 0.0027, 0.003, 0.0075, 0.011, 0.014, 0.014, 0.016, 0.023, 0.034, 0.035, 0.04).

11 Observations:

12 The data in Figures 2 and 4 show that it is possible to identify metabolic
13 differences in known groups; Figure 5 shows the reciprocal -- that the metabolic
14 profiles generated by coulometric array technology include sufficient information to
15 identify the group to which a sample belongs. Thus, metabolic profiles reflective of
16 long term DR may be used to group human samples, and the groups generated may in
17 turn reflect the samples' identity (e.g., women who later developed breast cancer vs
18 women who remained cancer free), and persons at high risk for development of
19 disease vs persons at low risk for development of disease).

20 There are five components linking the methodology of the present invention to
21 its utility. The first is the ability to identify an animal system in which disease
22 frequency is reproducibly reduced. This is accomplished by using the dietary
23 restricted rats, which have robustly increased longevity and decreased morbidity as
24 compared with their ad libitum fed counterparts. The second is a methodology that
25 enables us to capture serum components that differ between ad libitum and dietary
26 restricted rats. Direct evidence for the utility of our invention to complete this
27 component is shown in Figures 2B, 2C, 4 and 5. The third is based on the observation
28 that the metabolites identified are sufficient to group animals by caloric intake. This
29 is shown in Figure 5. The fourth component is based on the observation that at least
30 some of the markers (metabolites) identified in non-human species can be identified
31 in humans. This is true because of the overall similarity between the metabolism of

1 all mammals. Direct confirmation has been previously demonstrated by Milbury et al
2 in their comparative studies of the bear and humans {46}. Finally, the fifth
3 component is the ability of these markers, or subsets of them, to predict disease risk or
4 diagnose disease in humans. This follows from the general similarity of metabolism
5 between mammals, the strong association of many human diseases with caloric intake
6 (e.g., some cancers, type II diabetes, cardiovascular and cerebrovascular diseases), and
7 the established efficacy of DR against most forms of morbidity. Furthermore, the
8 method for determining which subsets of markers have utility includes generation and
9 verification of markers in animals coupled with testing these markers in human
10 populations using methods developed for human epidemiology. Intermediate steps,
11 such as testing multiple patterns in humans with defined nutritional intake, may be
12 used to facilitate and strengthen the approach.

13 Figure 5 shows the grouping of the sera samples from 6 and 12 month old rats
14 based on the metabolites that were identified as differing between 6-month old AL
15 and DR rats. The dendrograms in Figure 5 (panels A and B) were generated using the
16 hierarchical cluster analysis package from the Einsight data analysis package.
17 Hierarchical cluster analysis is a method of data analysis that emphasizes the natural
18 groupings of the data set. In contrast to analytical methods that emphasize
19 distinguishing differences between two groups, hierarchical cluster analysis uses
20 algorithms that reduce complex data sets to establish these groups without
21 preconceived divisions. In this dendrogram, relative similarity within the total study
22 population increases as one moves from right (0.0) to left (1.0, biochemical identity)
23 on the horizontal axis. The smaller the distance is from identity (left side) to the point
24 at which two samples (groups) are linked by a vertical line, the greater the relatedness
25 of the two samples (groups). Alternatively, the closer the split between two samples
26 is to the right of the figure, the greater the disparity between two samples or groups of
27 samples.

28 Additional analyses were also conducted using Eigenvector or principal
29 component analysis (PCA), which determines those analytes that contribute most
30 heavily to the separation of groups (panels C and D of Figure 5). In this type of
31 analysis, the two PCA components that were most significant at explaining the

1 variation in the database are termed PC 1 and 2, respectively. Relative mathematical
2 values were assigned to the two groups of analytes that best discriminate the data set
3 (PC-1 and PC-2, exact values are arbitrary). A scattergram then was plotted using the
4 PC-1 value for the X axis and the PC-2 value for the Y-axis. In the context of the
5 current invention, principal component (Eigenvector) analysis enabled us to identify
6 which of the multiple compounds that may differ between AL and DR animals were
7 the most useful for classification purposes. This analysis also gives a means of
8 estimating the consequences of removing different analytes from the profiles. This
9 type of analysis permits us readily to determine which analytes contribute the most to
10 our ability to distinguish members of one group from members of another (e.g.,
11 humans at high risk for developing a specific disease vs humans not at high risk for
12 developing that disease).

13 As shown in Figure 5, data of sufficient power can be generated such that both
14 hierarchical cluster analysis and principal component analysis were able to separate
15 the rat sera by dietary group in both the initial cohort of 6 month old rats (with 100%
16 accuracy, Figure 5A and 5C) and two independent cohorts of 12 and 18 month rats
17 (with >85% accuracy, Figures 5B and 5D. The initial group confirms a series of
18 markers that, by themselves, retain a sufficient fraction of the information present in
19 sera to enable one to correctly identify the origin of the samples. More importantly,
20 the studies in the two independent data sets reveal that the data is able to identify a
21 series of markers with sufficient power to correctly identify >85% of unknown,
22 independent samples. Equally successful separation was achieved at all three ages
23 regardless of whether all 22 markers were used or just the 6 markers that differed in
24 both 6 and 12 month samples. Misclassifications were limited to a small subset [2-4
25 rats] of the cohort, and were dependent on the markers used (6 or 22) and the exact
26 algorithms used to conduct the analysis.

27 **Serum Markers Distinguish AL and DR Rats**

28 The 22 serum metabolites identified as potential markers in 6 month old AL
29 and DR rats (Figure 4, left of vertical line) and the 6 markers shown to be replicable
30 in 6 and 12 month old rats (Figure 4, asterisks) were used to determine groupings of 3
31 sets of AL and DR rats (6, 12, and 18 months, 18 month data not shown). Rat

1 designations (e.g., A1) are consistent within age groups (vertically, e.g., A1 in Figs.
2 5A and 5C are the same rat, but A1 in Figs. 5A and 5B are not). Both hierarchical
3 cluster analysis (A,B) and principal component (Eigenvector) analysis (C,D) of the
4 data are shown. (A) Dendrogram of analysis of the sera from 14 6 month old rats.
5 All 22 compounds were used to determine the natural groupings, but similar results
6 were also obtained using only the 6 replicable markers. (B) Dendrogram of analysis
7 of the sera from 15 12 month old rats (independent test set). All 22 compounds were
8 used to determine the natural groupings. Similar results were also obtained using only
9 the 6 replicable markers and in samples from 18 month old rats. (C) Principal
10 component analysis of sera from the 14 6 month old rats using all 22 markers. Similar
11 results were also obtained using only the 6 replicable markers. (D) Principal
12 component analysis of the sera from the 15 12 month old rats in the independent test
13 set using the 6 replicable markers. Similar results were also obtained using all 22
14 markers as well as in samples from 18 month old rats. All analysis was based on first
15 pass data -- meaning that the HPLC data analysis software required no further training
16 and no human intervention to collect data of sufficient quality to distinguish AL and
17 DR rats.

18 The data presented in Figures 2, 4 and 5 demonstrate that the present invention
19 permits identification markers that reproducibly differ between AL and DR rats, and
20 that metabolite profiles based on these markers are sufficiently powerful to assign sera
21 samples into correct dietary groups by hierarchical cluster analysis and principal
22 component analysis with >85% accuracy -- even when these phenotypes may be
23 partially obscured by age-related and/or individual variation. Increasing the "N" will
24 readily increase the accuracy and power of these results by generating larger, and thus
25 more informative, training sets, and by increasing the signal-to-noise ratio by
26 removing noninformative metabolites from the profiles. Furthermore, building
27 extended databases using rats maintained on specifically modified feeding regimens
28 will enable one to parse out metabolites and metabolic profiles to increase power (e.g.,
29 one can identify markers that reflect a short term diet and distinguish those which
30 reflect a truly long term reduced caloric intake). Both of these sets of markers may
31 have utility for different uses. Finally, the data obtained can be analyzed by

1 univariate, multivariate, or pattern recognition based analyses, and that these analyses
2 may detect utility not seen with other analyses.

3 It thus appears that HPLC with coulometric-array detectors advantageously
4 may be employed to identify specific chemical markers, i.e. metabolites, sets of
5 metabolites, and/or metabolic profiles (detected in sera or other biological samples)
6 that separates AL from DR rats or other animals, and that such metabolites, sets of
7 metabolites, or metabolic profiles in turn may be used to diagnose or predict disease
8 states or future risks of diseases. Such diseases may include degenerative diseases
9 such as diabetes, in particular, type II diabetes, cardiovascular disease, stroke, heart
10 attack, cerebrovascular disease, and other diseases whose etiology has been
11 established to or hypothesized to (e.g., Alzheimer's {1}) be modified by diet or
12 nutrition, although utility in other diseases is also considered, including, neoplastic
13 and non-neoplastic diseases, such as breast cancer, colon cancer, pancreatic cancer,
14 lymphoma, prostate cancer and leukemia, neurological diseases, neurodegenerative
15 diseases, autoimmune diseases, endocrine diseases, renal disease, Huntington's
16 disease, Parkinson's disease, Lou Gehrig's disease, and the like, as well as sensitivity
17 to toxins, e.g. industrial and/or environmental toxins. Moreover, applying the
18 technique of the present invention to a larger number of samples will permit one to
19 observe greater number of chemical pattern characteristics, and to identify new
20 chemical patterns and/or new markers specific to particular diseases and/or sub-
21 clinical conditions that in the future may develop into a specific disease. In turn, this
22 may permit early intervention and thus possibly head off the development of the
23 disease. The invention also advantageously may be employed for diagnosing other
24 disease conditions, or sub-clinical conditions, i.e. before observable physical
25 manifestations, that in the future may develop into disease conditions. Similarly, in
26 addition to disease, the assessment of nutritive status may be useful as a medical test
27 under a variety of potential clinical settings, or in controlling epidemiological or
28 pharmaceutical testing, although other utilities are contemplated.

REFERENCES

1. Grant, WB, Dietary Links to Alzheimer's Disease, Alzheimer's Disease Review, 2, 42-55, 1997; see also Mark Mattson et al, _____, 1999.
2. Kristal, B.S. and BP Yu, Aging and its modulation by caloric restriction, in. B.P. Yu, Ed. Modulation of Aging Processes by Dietary Restriction, CRC Press, 1994. pp. 1-35.
3. Weindruch, R. and R. Walford, The Retardation of Aging and Disease by Dietary Restriction, Charles C. Thomas, St. Louis, 1988.
4. Yu, B.P., Aging and oxidative stress: modulation by dietary restriction, Free Rad. Biol. Med., 21:651-668, 1996.
5. McCay, C.M., Cellulose in the diet of rats and mice, J. Nutr., 435-447, 1935.
6. Maeda, H., CA Gleister, EJ Masoro, I Murata, CA McMahan, BP Yu, Nutritional influences on aging of Fisher 344 rats: II Pathology, J. Gerontol., 40:671-688, 1985.
7. Carlson, AJ, and F Hoelzel, Apparent prolongation of the life span of rats by intermittent fasting, J. Nutr., 31:363-375, 1946.
8. Goodrick, C.L., DK Ingram, MA Reynolds, JR Freeman, and N. Cider, Effects of intermittent feeding upon body weight and lifespan in inbred mice: Interaction of genotype and age, Mech Age. Dev., 55:69-87, 1990.
9. Cheney, KE, RK Liu, GS Smith, RE Leung, MR Mickey, and RL Walford, Survival patterns in C57BL/6J mice subjected to undernutrition, Exp. Gerontol., 15:237-258, 1980.
10. Ross, MH, and G. Bras, Food preference and length of life, Science, 190:165-167, 1970.
11. Weindruch, R, RL Walford, Dietary restriction in mice beginning at 1 year of age: Effect on life-span and spontaneous cancer incidence, Science, 215:1415-1418, 1982.
12. Nolen, GA, Effect of various restricted dietary regimens on the growth, health, and longevity of albino rats, J. Nutr., 102:1477-1494, 1972.

- 1 13. Tannenbaum, A., The dependence of tumor formation on the composition of
2 the calorie-restricted diet as well as on the degree of restriction, *Cancer Res.*,
3 5:616-625, 1945.
- 4 14. Iwasaki, K., CA Gleister, EJ Masoro, CA McMahan, E-J Seo, and BP Yu, The
5 influence of dietary protein source on longevity and age-related disease
6 processes of Fischer rats, *J. Gerontol.*, 43:B5-B12, 1988.
- 7 15. Iwasaki, K., CA Gleister, EJ Masoro, CA McMahan, E-J Seo, BP Yu,
8 Influence of the restriction of individual dietary components on longevity and
9 age-related disease of Fischer Rats: the fat component and the mineral
10 component, *J. Gerontol.*, 43:B13-21, 1988.
- 11 16. Stucklikova, E., M. Juricova-Horakova, Z. Deyl, New aspects of the dietary
12 effect of life prolongation in rodents. What is the role of obesity in aging?
13 *Exp. Gerontol.*, 10:141-144, 1975.
- 14 17. Lane, MA, DJ Baer, WV Rumpler, R Weindruch, DK Ingram, EM Tilmont,
15 RG Cutler, GS Roth, Calorie restriction lowers body temperature in rhesus
16 monkeys, consistent with a postulated anti-aging mechanism in rodents, *Proc.*
17 *Natl. Acad. Sci.*, 93:4159-64, 1996.
- 18 18. Kemnitz, JW, EB Roecker, R Weindruch, DF Elson, ST Baum, RN Bergman,
19 Dietary restriction increases insulin sensitivity and lowers blood glucose in
20 rhesus monkeys, *Am. J. Physiol.*, 266:E540-7, 1994.
- 21 19. Hansen, BC, HK Ortmeyer, NL Bodkin, Prevention of obesity in middle-aged
22 monkeys: Food intake during body weight clamp, *Obesity Res.*, 3 Suppl
23 2:199S-204S, 1995.
- 24 20. Bodkin, NL, HK Ortmeyer, BC Hansen, Long-term dietary restriction in older-
25 aged rhesus monkeys: effects on insulin resistance, *J. Gerontol.*, 50:B142-147,
26 1995.
- 27 21. Lane, MA, SS Ball, DK Ingram, RG Cutler, J. Engel, V Read, GS Roth, Diet
28 restriction in rhesus monkeys lowers fasting and glucose-stimulated
29 glucoregulatory end points, *Am. J. Physiol.*, 268:E941-8, 1995.
- 30 22. Walford, RL, L Weber, S Panov, Caloric restriction and aging as viewed from
31 Biosphere 2, *Receptor*, 5:29-33, 1995.

- 1 23. Walford, RL, SB Harris, MW Gunion, The calorically restricted low-fat
2 nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total
3 leukocyte count, cholesterol, and blood pressure in humans, *Proc. Natl. Acad.*
4 *Sci.*, 89:11533-537, 1992.
- 5 24. Berg, BN, and HS Simms, Nutrition and longevity in the rat. II. Longevity and
6 onset of disease with different levels of food intake, *J. Nutr.*, 71:255-263, 1960
- 7 25. McCay, C.M., G Sperling, LL Barnes, Growth, aging, chronic diseases and life
8 span in rats, *Arch Biochem. Biophys.*, 2:469-479, 1943.
- 9 26. Fernandes, GP, EJ Friend, EJ Yunis, RA Good, Influence of DR on
10 immunologic function and renal disease in (NZB x NZW) F1 mice, *Proc. Natl.*
11 *Acad. Sci.*, 75:1500-1504, 1978.
- 12 27. Mark, DA, DR Alonso, F Quimby, HT Thaler, YT Kim, G Fernandes, RA
13 Good, ME Weksler, Effect of nutrition on disease and life span, I. Immune
14 responses, cardiovascular pathology, and lifespan in MRL mice, *Am. J.*
15 *Pathol.*, 117:110-124, 1984.
- 16 28. Lloyd, T., Food restriction increases life-span of hypertension animals, *Life*
17 *Sci.*, 34:401-407, 1984.
- 18 29. Russell, JC, DG Koeslag, V Manickavel, RM Amy, PJ Dolphin, Effects of
19 advancing age and severe food restriction on pathological processes in the
20 insulin resistant JCR:LA-corpulent rat, *Diabetes Res.*, 15:53, 1990.
- 21 30. Shimokawa, I, and Y Higami, Effect of dietary restriction on pathological
22 processes, in. B.P. Yu, Ed. *Modulation of Aging Processes by Dietary*
23 *Restriction*, CRC Press, 1994. pp. 247-266.
- 24 31. Weindruch, R., RJ Walford, S Fliegel, D Guthrie, The retardation of aging in
25 mice by dietary restriction: longevity, cancer, immunity and lifetime energy
26 intake, *J. Nutr.*, 116:641-654, 1986.
- 27 32. Fernandes, G., B. Chandrasekar, DA Troyer, JT Venkatraman, RA Good,
28 Dietary lipids and calorie restriction affect mammary tumor incidence and
29 gene expression in mouse mammary tumor virus/v-Ha-ras transgenic mice,
30 *Proc. Natl. Acad. Sci.*, 92:6494-6498, 1995.

- 1 33. Kritchevsky, D., MM Weber, DM Klurfeld, Dietary fat versus caloric content
2 in initiation and promotion of 7,12-dimethylbenz(a)anthracene-induced
3 mammary tumorigenesis in rats, *Cancer Res.*, 44:3174-3177, 1984.
- 4 34. Klurfeld, DM, MM Weber, D Kritchevsky, Inhibition of chemically induced
5 mammary and colon tumor promotion by caloric restriction in rats fed
6 increased dietary fat, *Cancer Res.*, 47:2759-2762, 1987.
- 7 35. Sinha, DK, RL Gebhard, JE Pazik, Inhibition of mammary carcinogenesis in
8 rats by dietary restriction, *Cancer Lett.*, 40:133-141, 1988.
- 9 36. Tucker, MJ, The effect of long-term food restriction on tumours in rodents,
10 *Int. J. Cancer*, 23:803-807, 1987.
- 11 37. Yu, BP, Food restriction research: past and present status, *Rev. Biol. Res* in
12 *Aging*, 4:349-371, 1990.
- 13 38. Sabatino, F, EJ Masoro, CA McMahan, RW Kuhn, Assessment of the role of
14 the glucocorticoid system in aging processes and in the action of food
15 restriction, *J. Gerontol.*, 46:B171-179, 1991.
- 16 39. Holehan, AM, BJ Merry, The control of puberty in the dietary restricted
17 female rat, *Mech. Age. Dev.*, 32:179-191.
- 18 40. Green, G, D Guan, J Morisset, A Spannagel, E Paul, Preservation of exocrine
19 pancreatic function in food-restricted rats, *Pancreas*, 8:757, 1993
- 20 41. Herlihy, JT, C. Stacy, HA Bertrand, Long term food restriction depresses
21 serum thyroid hormone concentrations in the rat, *Mech. Age. Dev.*, 53:9, 1990
- 22 42. Masoro, EJ, RJM McCarter, MS Katz, CA MacMahan, Dietary restriction
23 alters characteristics of glucose fuel use, *J. Gerontol.*, 47:B202-208, 1992.
- 24 43. Taylor, A, RD Lipman, J Jahngen-Hodge, V Palmer, D Smith, N Padhye, GE
25 Dallal, DE Cyr, E. Laxman, D Shepard, Dietary calorie restriction in the
26 Emory mouse: effects on lifespan, eye lens cataract prevalence and
27 progression, levels of ascorbate, glutathione, glucose, and glycohemoglobin,
28 tail collagen breaktime, DNA and RNA oxidation, skin integrity, fecundity,
29 and cancer, *Mech. Age. Dev.*, 79:33-57, 1995.

- 1 44. Taylor, A., J Jahngen-Hodge, DE Smith, VJ Palmer GE Dallal, RD Lipman, N
2 Padhye, B Frei, Dietary restriction delays cataract and reduces ascorbate levels
3 in Emory mice, Exp. Eye Res., 61:55-62, 1995.
- 4 45. Harris, SB, MW Gunion, MJ Rosenthal, RL Walford, Serum glucose, glucose
5 tolerance, corticosterone and free fatty acids during aging in energy restricted
6 mice, Mech. Age. Dev., 73:209-21, 1994.
- 7 46. The Tenth International Conference on Bear Research and Management of
8 July 16-20, 1995 at Fairbanks, Alaska
- 9

1 CLAIMS

- 2 1. In a method for diagnosing and/or predicting disorders in which
3 biological samples are analyzed to generate frequency distribution patterns
4 representative of molecular constituents of said samples, the improvement which
5 comprises comparing frequency distribution patterns of constituents of samples of ad
6 libitum-fed and dietary-restricted individuals.
- 7 2. A method according to claim 1, wherein said samples comprise body
8 fluids.
- 9 3. A method according to claim 2, wherein said body fluids are selected
10 from the group consisting of serum, plasma, platelets, saliva and urine.
- 11 4. A method according to claim 1, wherein said disorder is selected from
12 the group consisting of neoplastic or non-neoplastic disease, cardiovascular or
13 cerebrovascular disease, renal disease, autoimmune disease, neurological or
14 neurogenerative disease, endocrine disease, and diabetes.
- 15 5. A method according to claim 1, wherein said disorder is selected from
16 the group consisting of breast cancer, colon cancer, pancreatic cancer, lymphoma,
17 prostrate cancer and leukemia.
- 18 6. A method according to claim 1, wherein said disorder comprises
19 glomerulonephritis.
- 20 7. A method according to claim 1, wherein said disorder comprises
21 periarateris.
- 22 8. A method according to claim 1, wherein said disorder is selected from
23 the group consisting of myocardial degeneration, heart disease and stroke.
- 24 9. A method according to claim 1, wherein said disorder comprises
25 altherosclerosis.
- 26 10. A method according to claim 1, wherein said disorder comprises
27 pituitary adnoma.
- 28 11. A method according to claim 1, wherein said disorder comprises type II
29 diabetes.
- 30 12. A method according to claim 1, wherein said disorder comprises
31 sensitivity to toxins.

- 1 13. A method according to claim 1, wherein said comparison is conducted
2 using univariat statistics.
- 3 14. A method according to claim 1, wherein said comparison is conducted
4 using multivariat statistics.
- 5 15. A method according to claim 1, wherein said comparison is conducted
6 using hierarchical cluster analysis.
- 7 16. A method according to claim 1, wherein said comparison is conducted
8 using principal component analysis.
- 9 17. A method according to claim 1, wherein said comparison is conducted
10 using pattern recognition algorithms to qualitatively and quantitatively identify
11 metabolic profiles and relationships.
- 12 18. A method according to claim 1, wherein said biological samples
13 comprise electrochemically active compounds, and including the steps of passing said
14 fluid samples sequentially through a liquid chromatographic column for achieving
15 time-space separation of the materials eluting from the column, and an
16 electrochemical detection apparatus whereby to generate electrochemical patterns of
17 said electrochemically active compounds.
- 18 19. A method according to claim 18, including the step of separating said
19 electrochemically active compounds by electrochemical characteristics in said
20 electrochemical detection apparatus.

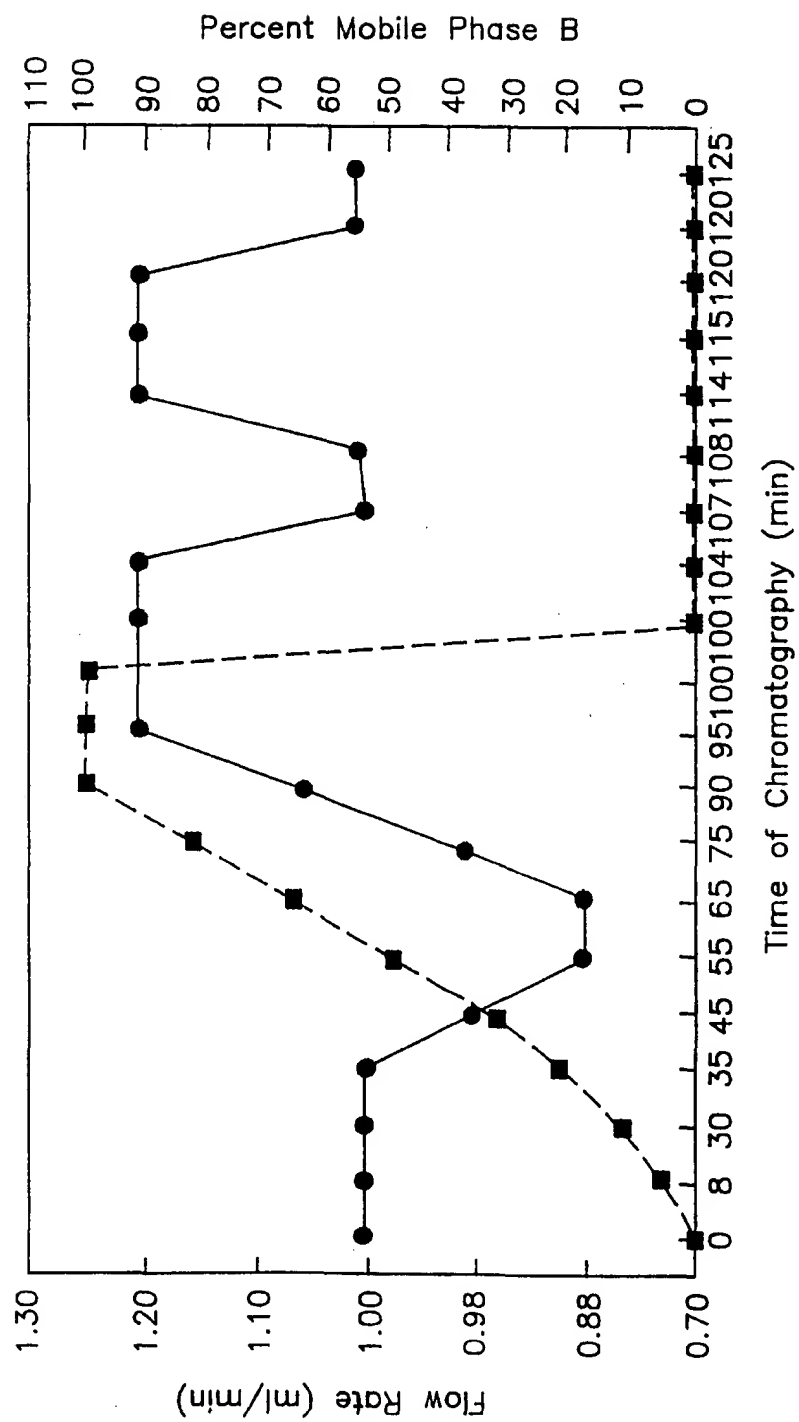


FIG. 1

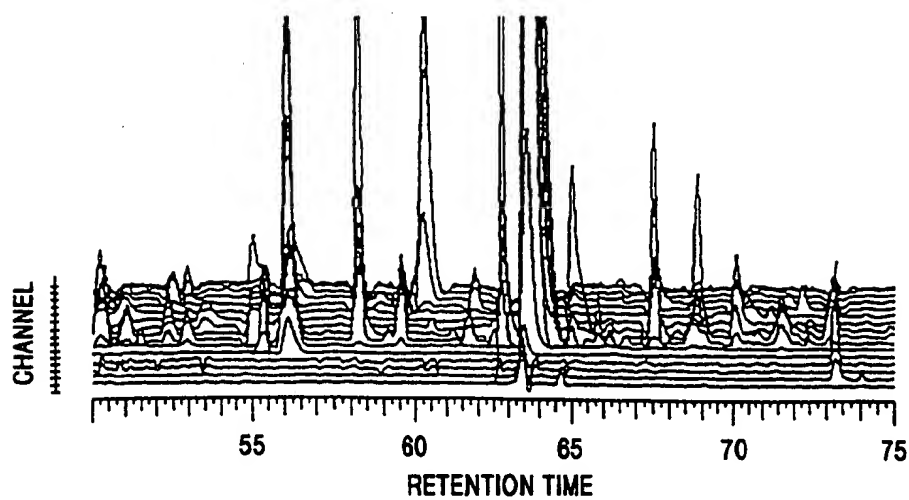


FIG. 2A

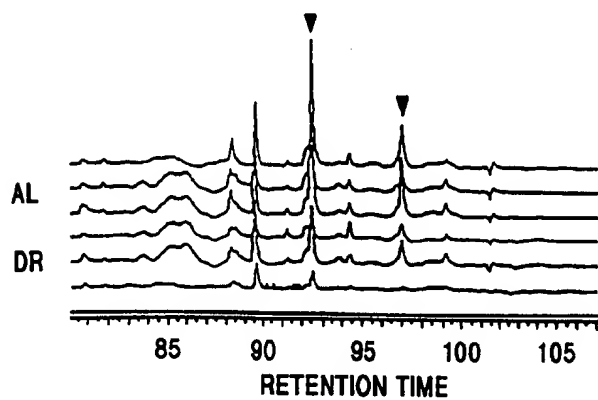


FIG. 2B

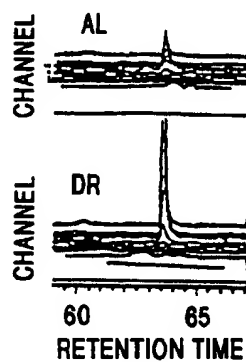
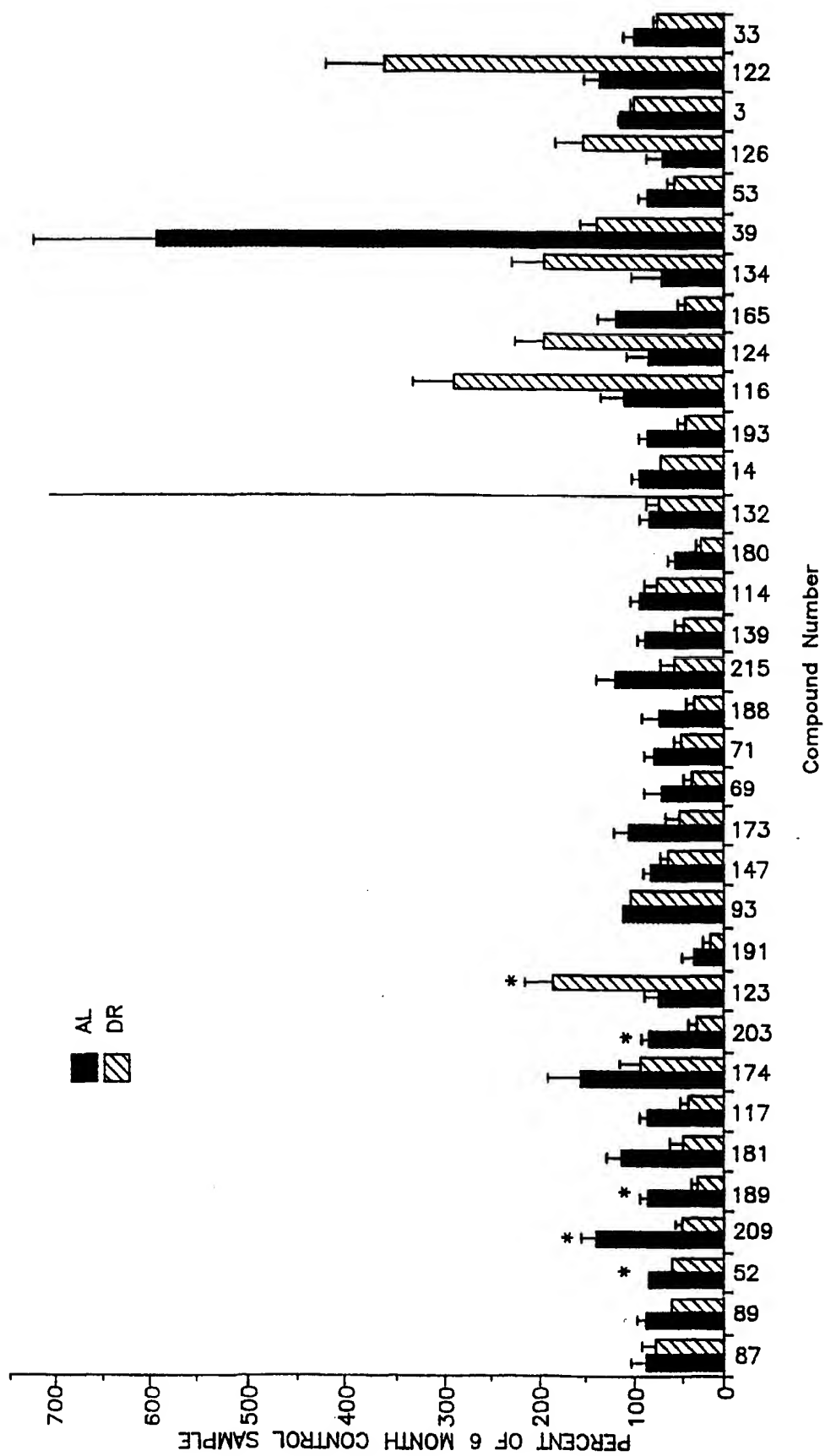


FIG. 2C

α -methyhistidine	ferulic acid
1-methylhistidine	glutathione
2-hydroxyphenylacetic acid	glutathione disulfide
3,4-dihydroxymandelic acid	guanine
3,3,5-triiodothyronine	homocamosine
3,4-dihydroxyphenylacetic acid	homogentisec acid
3,0-methyldopa	homovanillic acid
3-hydroxy-4-methylphenethylamine	homovanylyl alcohol
3-hydroxyanthranilic acid	homoveratic acid
3-hydroxykynurenine	hypoxanthine
3-hydroxymandelic acid	indole-3-lactic acid
3-hydroxyphenylacetic acid	indole-3-propionic acid
3-methoxy-4-hydroxyphenylglycol	indoleacetic acid
3-methoxytyramine	isatin
3-methylhistidine	isoproterenol
4-hydroxy-3-methylmandelic acid	kynurenine
4-hydroxybenzoic acid	levodopa
4-hydrocyphenylacetate	melatonin
4-hydrocyphenylacetate	metanephrine
4-O-methyldopamine	methionine
5-hydroxyindoleacetic acid	methoxamine
5-hydroxytryptophan	n-acetylserotonin
5-hydroxytryptophol	n-methylserotonin
5-methoxytryptamine	norepinephrine
5-methoxytryptophan	normetanephrine
5-methoxytryptophol	pyridoxal
5-methylcysteine	serotonin
6-hydroxymelatonin	tryptamine
7-methylguaninne	tryptophan
7-methylxanthine	tryptophol
acetylhistidine	tyramine
anserine	tyrosine
anthranillic acid	uric acid
ascorbic acid	vanillic acid
camosine	vanillylmandelic acid
cysteine	xanthine
dopamine	xanthosine
epinephrine	

FIG. 3



Compound Number

FIG. 4

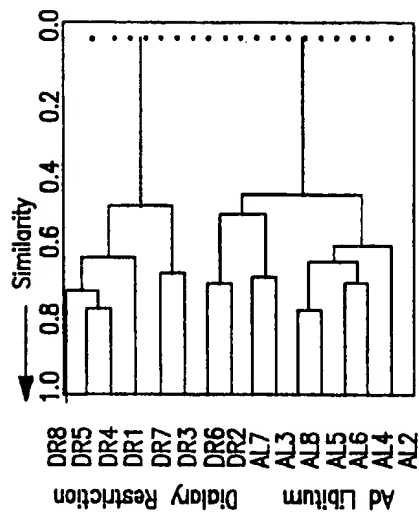


FIG. 5B

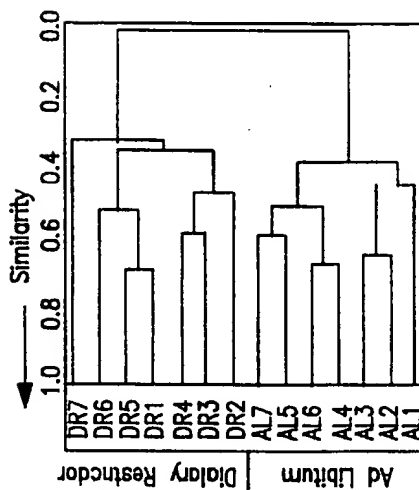


FIG. 5A

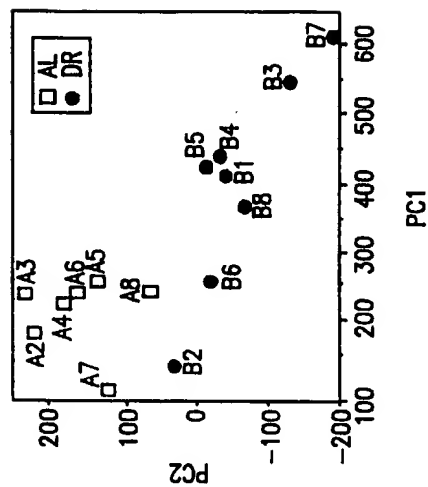


FIG. 5D

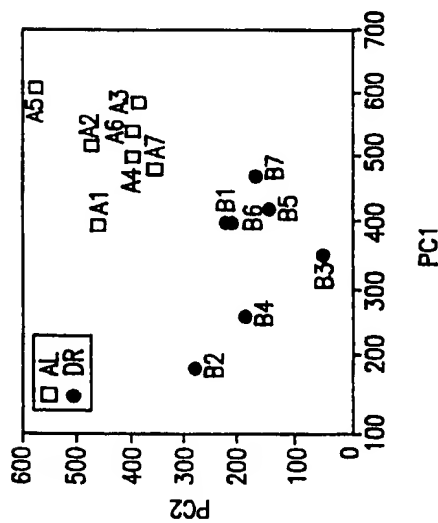


FIG. 5C

	Retention Time	Array Channel	Aprox. Oxidation Potential
Compound 52	26.37	10	530
Compound 55	27.98	9	460
Compound 69	35.08	16	950 or higher
Compound 87	46.83	16	950 or higher
Compound 89	49.72	8	390
Compound 93	51.92	8	390
Compound 114	59.81	16	950 or higher
Compound 117	62.16	7	320
Compound 123	63.30	16	950 or higher
Compound 132	67.24	14	810
Compound 139	69.78	11	600
Compound 147	72.18	8	390
Compound 173	81.82	9	460
Compound 174	83.88	6	250
Compound 180	88.61	13	740
Compound 181	88.57	10	530
Compound 188	92.24	10	530
Compound 189	92.43	9	460
Compound 191	93.79	5	180
Compound 203	96.99	8	390
Compound 209	98.53	6	250
Compound 215	101.64	10	530

FIG. 6

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US99/06762

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 31/00, 29/00, 7/38

US CL : 435/149, 63, 64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/149, 63, 64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database MEDLINE on STN Acc. No. 91248001, Granzotti et al. Nutritional Index in heart disease in childhood. Arquivos Brasileiros de cardiologia. December 1990, Vol. 55, No. 6, pages 371-373, see abstract.	1-19
A	Database MEDLINE on STN, AN 91108912, Liver function tests abnormalities in patients with inflammatory bowel disease receiving artificial nutrition: a perspective randomized study of total enteral nutrition versus total parenteral nutrition. Abad-Lacruz et al. November-December 1990. JPEN J. Parenteral and Enteral Nutrition, Vol. 14, No. 6, pages 618-621.	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 JUNE 1999

Date of mailing of the international search report

14 JUL 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06762

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE, SCISEARCH USPATFULL

search terms: diagnosis, disease or disorder, nutritional status, metabolic process, predict disorders